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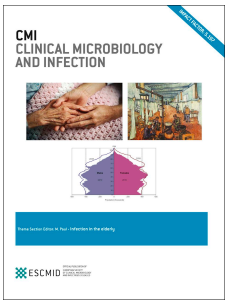

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A simple phenotypic method for screening of MCR-1-mediated colistin resistance

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Running title: Colistin-MAC test for phenotypic screening of MCR-1

Abstract

Objectives: To evaluate a novel method, named Colistin-MAC test, for phenotypic screening of acquired colistin-resistance mediated by transferable *mcr-1* resistance determinants, based on colistin MIC reduction in the presence of dipicolinic acid (DPA).

Methods: The Colistin-MAC test consists in a broth microdilution method, in which colistin MIC is tested in absence or presence of DPA (900 μg/mL). Overall, 74 colistin-resistant strains of Enterobacteriaceae (65 *Escherichia coli* and 9 of other species), including 61 strains carrying *mcr-1*-like genes and 13 strains negative for *mcr* genes, were evaluated with the Colistin-MAC test. The presence of *mcr-1*-like and *mcr-2*-like genes was assessed by Real-Time-PCR and end-point PCR. For 20 strains, Whole-Genome Sequencing data were also available.

Results: A ≥8-fold reduction of colistin MIC in the presence of DPA was observed with 59 *mcr-1*-positive strains, including 53 *E. coli* of clinical origin, three *E. coli* transconjugants carrying MCR-1-encoding plasmids, one *Enterobacter cloacae* complex, and two *Citrobacter* spp. Colistin MICs were unchanged, increased, or at most reduced by two-fold with the 13 *mcr*-negative colistin-resistant strains (nine *E. coli* and four *Klebsiella pneumoniae)*, but also with two *mcr-1*-like-positive *K. pneumoniae* strains.

Conclusions: The Colistin-MAC test could be a simple phenotypic test for presumptive identification of *mcr-1*-positive strains among isolates of colistin-resistant *E. coli*, based on a ≥8-fold reduction of colistin MIC in the presence of DPA. Evaluation of the test with a larger number of strains, species, and *mcr*-type resistance determinants would be of interest.

Keywords: DPA-colistin synergy, phenotypic screening, colistin resistance, *mcr-1,* Colistin-MAC test

Introduction

Polymyxins have lately regained a key role as last-resort antibiotics for treatment of infections caused by multidrug-resistant and extremely drug-resistant Gram-negative pathogens [1].

In Enterobacteriaceae, acquired resistance to polymyxins is mostly mediated by modification of the Lipid A target (by addition of 4-amino-4-deoxy-l-arabinose and/or phosphoethanolamine residues) that impair polymyxin binding [2]. Target modification can be dependent on chromosomal mutations upregulating the endogenous lipopolysaccharide modification systems, or by exogenous phosphoethanolamine transferases encoded by acquired mobilized colistin resistance (*mcr*) genes. Discovery of the latter mechanisms has raised considerable concern in view of their potential for spreading among clinical pathogens [3]. Indeed, after the first description [4], *mcr-1* and variants thereof have been detected worldwide [5], while additional types of *mcr* genes (e. g. *mcr*-2, *mcr*-3 and *mcr*-4) have recently been identified [6-8].

Since MCR-1 is a zinc enzyme [9], exposure to chelators could reduce colistin resistance in MCR-1-producing strains. Indeed, EDTA was recently reported to potentiate the activity of colistin against MCR-1-producing strains [9].

Here we describe a novel test (named Colistin-MAC test), based on colistin MIC reduction in the presence of dipicolinic acid (DPA), that could be useful for phenotypic screening of *mcr-1*-positive colistin-resistant *E. coli* strains.

Methods

The Colistin-MAC test is based on colistin MIC testing, in absence or presence of DPA, in a broth microdilution format according to the CLSI standard [10]. Colistin sulphate (Sigma-Aldrich, St Louis, MO, USA) concentrations tested ranged from 0.125 to 8.0 μg/mL, alone or in combination with DPA at a fixed concentration of 900 μg/mL. DPA was chosen since it was reported to have greater selectivity for zinc ions [11], and exhibited a better performance than EDTA in phenotypic

assays for detection of zinc-β-lactamases [12]. The DPA (Sigma-Aldrich) stock solution was prepared in dimethyl sulfoxide (DMSO) (SERVA, Heidelberg, Germany) at a concentration of 100 mg/mL and stored at -20°C. Cation-adjusted Mueller Hinton broth (MHB) (Becton Dickinson, Milan, Italy) was used as the medium for susceptibility testing. MHB containing DPA and DMSO at final concentrations of 900 μg/mL and 0.9% (v/v), respectively, was used for growth control. Susceptibility testing was carried out in 96-well microtiter plates (Sarstedt, Nümbrecht, Germany). Results were recorded after 20 hours of incubation at 35±2°C. Susceptibility to colistin was interpreted according to the EUCAST clinical breakpoints (≤ 2 μg/mL) [13]. All tests were carried out in duplicate. The two repetitions were considered concordant when a ≥8-fold reduction or a ≤2-fold reduction of colistin MIC in the presence of DPA was observed in both replicates. In case of discordant results, a third replicate was carried out and the modal value was considered.

The Colistin-MAC test was evaluated with 74 colistin-resistant strains from our Laboratory collection (Table S1), including: i) 61 strains carrying *mcr-1*-like genes, in which the genes had been detected by PCR or Whole-Genome Sequencing (WGS); ii) 13 strains in which the absence of *mcr-1*-like and *mcr-2* genes had been confirmed by RT-PCR and end-point PCR (Table S2); in nine of these strains the absence of all known *mcr* genes *mcr-1* to *mcr-4* had also been confirmed by WGS analysis. Most strains were *E. coli* (N=65), and a few belonged to other enterobacterial species including *Klebsiella pneumoniae* (N=6), *Citrobacter braakii* (N=1), *Citrobacter freundii* complex (N=1) and *Enterobacter cloacae* complex (N=1). The tested strains included 70 isolates of clinical origin (blood or urine cultures, or surveillance rectal swabs), one isolate of environmental origin, and three laboratory-derived *E. coli* J53 transconjugants harboring different *mcr-1*-carrying plasmids (Table S1). Some of the investigated strains have been previously reported, while others are part of an ongoing study. All *E. coli* strains were confirmed to be clonally unrelated by random amplification of polymorphic DNA PCR (Table S1). The *K. pneumoniae* strain carrying *mcr-1*-like gene was ST1 and presented a capsular *wzi-19* allele, while that carrying *mcr-1.2* was ST512 and presented a capsular *wzi-154* allele.

The effect of DPA in increasing colistin susceptibility of *mcr-1*-like positive strains was also tested in a disc-diffusion format [14], using colistin discs (10 μg, Thermo Scientific™ Oxoid™ , Waltham, MA USA) added with either 7.5 μL or 10 μL of the DPA stock solution (i. e. 750 or 1000 μg per disc, respectively) before placing the disc onto the inoculated medium.

Results

The tested strains exhibited a colistin MIC ranging from 4 to >8 μg/mL (Table 1), and all of them grew well in the presence of DPA and DMSO at the concentrations used in the test.

In presence of DPA at 900 μg/mL, all the 53 *mcr-1* positive *E. coli* strains exhibited a reduction of colistin MIC of at least an 8-fold dilution (range, 8 – ≥128 fold). A similar behavior was also observed with the three *E. coli* J53 transconjugants carrying different *mcr-1* plasmids, with the *mcr-1*-positive *Enterobacter cloacae* complex strain and with the two *mcr-1*-positive *Citrobacter* strains (Table 1). Discordant results between the two replicates were only observed with three *mcr-1* positive *E. coli* strains, which resulted in 4-fold reduction of colistin MIC in one of the two repetitions. In these cases, a third replicate yielded a >8-fold colistin MIC reduction (Table S1) and the median value was considered. Colistin MIC was increased, unchanged or at most decreased by a two-fold dilution with the *mcr*-negative *E. coli* and *K. pneumoniae* strains, but also with the two *mcr-1*-like positive *K. pneumoniae* strains (Table 1).

In the disc-diffusion format, no significant differences were detected in inhibition zones between *mcr-1*-positive and *mcr*-negative colistin-resistant strains (data not shown), probably due to the low and variable diffusibility of colistin from discs [15].

Discussion

The Colistin-MAC test described in this work could be a simple method for screening of *mcr-1*-positive strains among colistin-resistant *E. coli* and, possibly also of other species of Enterobacteriaceae. In fact, considering as a criterion for *mcr-1* positivity/negativity a ≥8-fold /≤2-fold reduction of colistin MIC in the presence of DPA, the test was able to correctly categorize all

the 65 colistin-resistant *mcr-1*-positive *E. coli* (including 62 clinical strains and three laboratory-derived transconjugants), and also a few *mcr-1*-positive strains of other species. The lack of inhibitory effect observed with the *mcr-1*-positive *K. pneumoniae* could be due to a reduced permeability to DPA and/or to the presence of additional unknown mechanisms of colistin resistance in those strains. We encountered only three results falling in the intermediate range (4-fold colistin MIC reduction by DPA), that were confirmed as positive (≥8-fold colistin MIC reduction) in two additional replicates. In case of an intermediate result, we suggest repeating the test and, if the intermediate result is confirmed, reporting the result as indeterminate.

This study was conceived as a preliminary proof of concept evaluation of the Colistin-MAC test and, as such, has a number of limitations including: i) the small sample size of investigated strains; ii) the limited number of investigated species, with a very small number of strains of species other than *E. coli*; iii) the small number of strains with (putative) chromosomal mechanisms of resistance, as negative controls; iv) the lack of strains carrying *mcr* genes other than *mcr-1*. The performance of this test should be confirmed with a larger collection of strains representative of different species and different colistin resistance mechanisms. The Colistin-MAC test has also some inherent limitations: i) the test is apparently not working with *K. pneumoniae*; ii) the execution requires the preparation of test-specific BMD panels, which can be labor-intensive and could increase costs in case of commercial production. In this perspective, it could be interesting to evaluate the performance of the test carried out with the addition of DPA directly to wells of a commercial panel.

Nevertheless, the possibility to presumptively detect *E. coli* strains with plasmid-mediated colistin resistance due to acquisition of *mcr-1* genes (which overall appear to be the most prevalent strains with transferable colistin resistance worldwide) with a test that can be set up also in basic laboratories not equipped with facilities for molecular testing, could be of remarkable importance for surveillance purposes, especially in low-income settings.

Transparency Declaration

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Table 1. Bacterial strains tested in this study, and colistin MIC values measured by broth microdilution in absence or presence of 900 mg/mL DPA (Colistin-MAC test). For a detailed description of tested strains and results see Table S1.

Species	Mechanism of Colistin resistance[a]	No. of strains	MIC Colistin (µg/mL) (median value)	MIC Colistin (µg/mL) + DPA 900 µg/ml (median value)	Fold of MIC reduction
Escherichia coli	*mcr-1*/ *mcr-1*-like	53	4 - >8 (8)	≤0.125 - 1 (≤0.125)	8 - ≥128
	mcr-NEG, n.d.	9	4 - 8 (8)	4 - >8 (8)	≈[b]
Escherichia coli J53AZI^{R} Transconjugants	*mcr-1*-like	3	4 (4)	≤0.125 - 0.5 (0.25)	8 - ≥32
Klebsiella pneumoniae	*mcr-1*-like/*mcr-1.2*	2	8 - >8	>8	≈[b]
	mcr-NEG PmrB mutant/inactivated *mgrB*/n.d.	4	>8	>8	≈[b]
Citrobacter braakii	*mcr-1*	1	8	0.5	16
Citrobacter freundii complex	*mcr-1*	1	8	≤0.125	≥64
Enterobacter cloacae complex	*mcr-1*	1	>8	≤0.125	≥128

[a] *mcr-1*-like indicates that the gene was amplified with primers for *mcr-1* but was not entirely sequenced; *mcr*-NEG indicates that the strain was negative for *mcr-1*-like and *mcr-2*-like genes as assessed by RT-PCR and end-point PCR. In some cases, WGS data confirmed negativity for all known *mcr*-genes; n.d., indicates that the colistin resistance mechanism remained not determined.

[b] ≈ indicates that colistin MIC, in presence of DPA, was increased, unchanged or at most decreased by a two-fold dilution.

Table S1. List of tested strains with mechanisms of colistin resistance, colistin MIC values in absence and presence of DPA, and references.

Table S2. Oligonucleotides and probes used for the RT-PCR and end-point-PCR for detection of *mcr-1-like* and *mcr-2-like* genes.